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Inhibitors of Protein Kinases and Phosphatases Alter Root Morphology and Disorganize Cortical Microtubules[1]

Tobias I. Baskin* and Jan E. Wilson

Division of Biological Sciences, University of Missouri, Columbia, Missouri 65211

To investigate molecular mechanisms controlling plant morphogenesis, we examined the morphology of primary roots of *Arabidopsis thaliana* and the organization of cortical microtubules in response to inhibitors of serine/threonine protein phosphatases and kinases. We found that cantharidin, an inhibitor of types 1 and 2A protein phosphatases, as previously reported for okadaic acid and calyculin A (R.D. Smith, J.E. Wilson, J.C. Walker, T.I. Baskin [1994] Planta 194: 516–524), inhibited elongation and stimulated radial expansion. Of the protein kinase inhibitors tested, chelerythrine, 6-dimethylaminopurine, H-89, K252a, ML-9, and staurosporine all inhibited elongation, but only staurosporine appreciably stimulated radial expansion. To determine the basis for the root swelling, we examined cortical microtubules in semithin sections of material embedded in butyl-methyl-methacrylate. Chelerythrine and 100 nM okadaic acid, which inhibited elongation without causing swelling, did not change the appearance of cortical arrays, but calyculin A, cantharidin, and staurosporine, which caused swelling, disorganized cortical microtubules. The stability of the microtubules in the aberrant arrays was not detectably different from those in control arrays, as judged by similar sensitivity to depolymerization by cold or oryzalin. These results identify protein phosphorylation and dephosphorylation as requirements in one or more steps that organize the cortical array of microtubules.

A plant cell in interphase usually has an array of microtubules, called the cortical array, just beneath its plasma membrane. The cortical array is highly ordered, comprising a large number of partly overlapping but mostly parallel microtubules (Gunning and Hardham, 1982; Cyr, 1994; Shibaoka, 1994). In cells expanding faster in one direction than in another, i.e. expanding anisotropically, the cortical array is organized so that most of the microtubules are perpendicular to the axis of most rapid expansion. When the array is removed, e.g. by treating plants with compounds such as oryzalin that cause the depolymerization of microtubules, morphology is disrupted (Hess, 1982; Vaughn and Lehnen, 1991). Without cortical microtubules, the normal highly anisotropic expansion of cells is replaced by isotropic expansion, which leads to the swollen form of the cell or organ. One exception is tip-growing cells, such as root hairs or pollen tubes, in which the role of microtubules seems unrelated to the polarity of expansion (Emons et al., 1992). Nevertheless, for a wide variety of plant and algal taxa and in diverse cell types, the cortical array is required for morphogenesis.

Despite the importance of the cortical microtubules for morphogenesis, it is not known how this array is organized. Microtubule arrays in animal cells are organized by virtue of the activity of microtubule-organizing centers, such as centrosomes or basal bodies (Mazia, 1984). These organizing centers provide preformed templates onto which tubulin can polymerize, overcoming the barrier for spontaneous nucleation and specifying the direction of microtubule growth. In plants, although templates of this type probably exist for organizing the radial array of microtubules emanating from the nucleus (Lambert, 1995), such templates have not been found to organize cortical microtubules. The ends of cortical microtubules generally lack the ultrastructural appearance that is typical of organizing material (Gunning and Hardham, 1982). Also, experiments designed to reveal such templates have provided evidence of their absence. These experiments involve a brief treatment with a microtubule inhibitor, sufficient to remove essentially all cortical microtubules, followed by washing out the drug and observing the re-forming cortical array at different times thereafter. Invariably, the array reappears randomly organized and only attains its typical alignment gradually over the course of a few hours (Cyr, 1994; Cyr and Palevitz, 1995; Lambert, 1995). If the array were organized by a preformed template, one would expect it to reappear with normal organization, which is exactly what happens when comparable experiments are done on the microtubule arrays of animal cells that emanate from centrosomes (Brinkley, 1985).

If the cortical array is not organized by a mechanism based on patterned nucleation, then the alternatives require that microtubules are differentially affected at different angles; however, the means whereby this is achieved is wholly unknown. Microtubule behavior in animal cells is known to be sensitive to protein phosphorylation; for example, phosphorylation increases microtubule dynamics and helps to reorganize microtubule arrays at the start of prophase (Verde et al., 1990; Sheldon and Wadsworth, 1996). Likewise, in plant cells microtubule behavior is thought to be influenced by protein phosphorylation (Cyr and Palevitz, 1995; Lambert, 1995). For example, treatment of suspension-culture cells with kinase inhibitors changes

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* Corresponding author; e-mail baskin@biosci.mbp.missouri.edu; fax 1–573–882–0123.

Abbreviations: DAPI, 4′, 6-diamidino-2-phenylindole; DMAP, 6-dimethylaminopurine.

the stability of microtubules to cold (Mizuno, 1992), and injection of cells in prophase with maturation-promoting factor purified from plants and containing activated $p34^{cdc2}$ kinase leads to a rapid depolymerization of the preprophase band (Hush et al., 1996). However, few studies have examined the role of protein phosphorylation for organizing cortical microtubules.

In earlier work, treatment of Arabidopsis seedlings with either of two inhibitors of Ser/Thr protein phosphatases (okadaic acid or calyculin) was shown to disrupt root morphology, suggesting that these compounds interfered with the function of cortical microtubules (Smith et al., 1994). In this study we extended that work to determine whether similar effects are also obtained by protein kinase inhibitors and to examine the effects of these inhibitors on the cortical array. We report that, like the phosphatase inhibitors, a kinase inhibitor disrupts root morphology, and in addition, in the cells of roots treated with either type of inhibitor, the microtubules of the cortical array become considerably disorganized. These results identify protein phosphorylation and dephosphorylation as part of the molecular mechanism that organizes the cortical array of microtubules.

MATERIALS AND METHODS

Inhibitors

The following lists the compounds used, their source (unless from L.C. Laboratories, Woburn, MA), the solvent used to make stock solutions if other than DMSO, and the maximum stock concentration. KN62 [1-[N,Obis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenyl piperizine], 10 mM; H-89 [*N*-[2-(*p*-bromocinnamylami- no)ethyl]-5-isoquinolinesulfonamide], water, 2 mM; calyculin A, 1 mM; cantharidin (gift of Prof. James Carrel, University of Missouri, Columbia), 100 mM; chelerythrine, water, 5.2 mM; ML-9 [1-(5-chloronaphthalene-1-sulfonyl)-homopiperizine], water, 3 mM; deltamethrin, 2 mM; K252a, 1 mM; KT5720, 1.86 mM; KT 5823, 2 mM; microcystin LR, 3 mM; okadaic acid, 1 mM; staurosporine, 2 mM; and tautomycin, 1 mM.

Plant Growth and Inhibitor Treatments

Seeds of *Arabidopsis thaliana* L. (Heynh), ecotype Columbia, were stored at 4°C, and at day 0 they were surface-sterilized and plated on agar-solidified, modified Hoagland solution with the following composition: 3% Suc, 1.2% agar, 4 mM KNO_3, 1 mM $Ca(NO_3)_2$, 0.3 mM $MgSO_4$, 2 mM KH_2PO_4, 89 μM iron citrate, 46.3 μM H_3BO_3, 9.1 μM $MnCl_2$, 0.77 μM $ZnSO_4$, 0.31 μM $CuSO_4$, and 0.11 μM MoO_3. Plates with seeds were placed vertically in a growth chamber with constant light (80 μmol m^{-2} s^{-1}) and temperature (20°C). Light from 40-W warm-white fluorescent bulbs and 25-W incandescent bulbs was filtered through a sheet of yellow acrylic (Plexiglas J2208, Cope Plastic, St. Louis, MO). Yellow filters were used to remove UV and blue wavelengths, which can drive deleterious photochemical reactions in plant growth media (Hangarter and Stasinopoulos, 1991). Experiments under white light were done in a comparable chamber without the yellow filter.

Seven days after plating, seedlings were transferred to freshly prepared plates containing the inhibitors, and the back of the plastic plate was scored at the position of the root tip. Plates were returned to the growth chamber and the position of the root tip was marked on the plastic again at 24 h. Forty-eight hours after the seedlings were transplanted, plates were photocopied at 1.5 times their original size. Root elongation during the preceding day was measured with a digitizing tablet as the distance along the root from the tip to the second mark. Root diameter was measured by placing a coverslip over the roots directly on the agar surface wetted by a few drops of an aqueous 0.01% (w/v) Triton X-100 solution. Mounted roots were viewed through a compound microscope at low magnification. In some experiments diameters were measured nondestructively after 24 h of treatment by viewing unmounted roots in "pseudo" dark-field by means of a large phase ring in the condenser. Diameters were measured with a video digitizer (Image 1/AT, Universal Imaging, West Chester, PA). For roots with conspicuous swellings, the diameter was measured at its apparent maximum; for control roots and others without apparent swelling, diameters were measured at the region of root hair initiation. Finally, for those treatments that inhibited root hair initiation, root diameter was measured just proximal of the zone of cell elongation. For these experiments three plates were made for each concentration, and 10 seedlings were transferred to each plate. Root elongation rates were measured for all three plates, and diameter was measured for two plates.

To test the stability of microtubules in the inhibitor-treated roots, seedlings were transplanted onto control or inhibitor plates for 15 h. Then, for experiments with oryzalin, roots were rinsed briefly in an aqueous solution of 1 μM oryzalin and seedlings were transplanted onto plates containing 1 μM oryzalin (or DMSO) and fixed as described below after 15 or 30 min. For experiments with cold, roots were rinsed briefly in ice-cold nutrient medium, and seedlings were transplanted onto ice-cold plates containing inhibitors (or DMSO) and fixed after 15 min.

Localization of Microtubules

Microtubules were localized in methacrylate sections using the method of Baskin et al. (1992, 1996) and that described here. Seedlings were fixed for 2 h at room temperature in 4% paraformaldehyde, 50 mM Pipes (pH 7.0), and 1 mM $CaCl_2$ by pouring the fixative solution directly onto the plates. For seedlings exposed to cold, ice-cold fixative was used and replaced by fresh, room-temperature fixative after 5 min. After three 10-min rinses in the above solution minus the fixative, about 3-mm apical root segments were encased in 2% (w/v in water) low gelling temperature agarose (type VII, Sigma). However, we found that straighter roots resulted if, instead of agarose, roots were held between two Formvar films on wire loops. For this method, 36-gauge copper wire was bent into a loop about 4 mm in diameter and coated with Formvar (0.25% in ethylene dichloride); then, the root tip was placed on the Formvar film, the overhanging root was cut away, and a second film of Formvar was added. The second film kept

the root from coming loose during dehydration. Samples were dehydrated in a graded ethanol series for 15 to 30 min per step. The ethanol series did not contain DTT (Baskin et al., 1996). Samples were infiltrated with 25, 50, and 75% resin in ethanol, for 30 min per step, and then three changes of 100% resin, for several hours per step, except that the second or third step continued overnight. From 50% ethanol through embedment, samples were kept at −20°C. Resin comprised 80% butyl-methacrylate, 20% methyl-methacrylate (Aldrich), 0.5% benzoinethylether (Aldrich), and 10 mM DTT (Baskin et al., 1992).

For embedment, agarose pellets or the wire loops with stems removed were placed individually in capsules manufactured with flat bottoms for embedding (TAAB Laboratories, Aldermaston, Berkshire, UK). Resin was polymerized under long-wavelength UV light at 4°C. Capsules were placed 8 cm beneath a 15-W source for 4 h. The embedded wire can be readily cut with a double-edged razor, so trimming the blocks is not impeded. Dry sections (1.75 μm) were cut on an ultramicrotome, placed in droplets of water, and affixed to slides coated with 3-aminopropyltriethoxy silane by heating briefly on a slide warmer for 2 to 5 min at 60°C (Angerer and Angerer, 1991). Twenty to 30 sections were collected from four to six roots per treatment.

For immunocytochemistry, sections were extracted in acetone (10 min) and then immediately hydrated in PBS containing 0.05% Tween 20 (PBS-Tween). Sections were rinsed in 0.1% Tween 20 in PBS, incubated in primary antibody (2 h at 37°C), rinsed in PBS-Tween (3 × 10 min), incubated in secondary antibody (2 h at 37°C), rinsed in PBS-Tween (3 × 10 min), with the second rinse also containing 1 μg mL^{-1} DAPI, and mounted in a commercial antifading reagent (Vectashield, Vector Laboratories, Burlingame, CA). Antibodies used were as follows: monoclonal anti-α-tubulin raised against sea urchin axonemes (B-5-1-2, Sigma) and used 1:1000; and as a secondary antibody, goat anti-mouse Fab fragments conjugated with Cy3 (Jackson Immuno-Research Laboratories, West Grove, PA) used at 1:200. Fluorescence microscopy was done with conventional epifluorescence (Zeiss Axioplan), and fluorescence from Cy3 was observed using the standard rhodamine filter cube.

To score mitotic index, sections were examined with UV excitation to excite DAPI fluorescence. The field iris for the epi-illumination was closed by about 40%, to the same position in all cases, the section was placed with the quiescent center at one edge of the field, and the image then encompassed the apical three-fourths of the zone of cell division. All nuclei were counted except those in root cap lineages, and all nuclei with clearly condensed chromosomes were counted as mitotic, which thus includes some late prophase and early telophase stages, as well as all prometaphase, metaphase, and anaphase stages.

RESULTS

Effects of Protein Phosphatase and Kinase Inhibitors on Root Morphology

To study the effects of various protein phosphatase and kinase inhibitors on root morphology, we grew Arabidopsis seedlings for 1 week on control medium, transplanted them onto plates with known concentrations of inhibitors, and measured elongation daily and maximum root diameter at the end of 2 d. The maximum diameter of untreated roots does not change appreciably over this period, so a measurement of initial root diameter is not needed. Several tested compounds, up to doses many orders of magnitude higher than the K_i for their chief target, were essentially inert in our system (Table I).

Three protein phosphatase inhibitors and six kinase inhibitors were effective (Fig. 1). The three phosphatase inhibitors, okadaic acid, calyculin, and cantharidin, all inhibited root elongation and stimulated radial expansion. Calyculin began to slow elongation and increase root diameter at the same dose (3 nM), whereas cantharidin and okadaic acid inhibited elongation at lower concentrations than they stimulated radial expansion (Fig. 1, A–C). For example, at 100 nM okadaic acid, elongation was reduced by 50%, but the diameter was not significantly increased (Fig. 1A).

The data for okadaic acid (Fig. 1A) are similar to those previously reported, but the response shown here for calyculin (Fig. 1B) is about 1 order of magnitude more sensitive (Smith et al., 1994). The difference in sensitivity occurred probably because calyculin is broken down in white light, as used by Smith et al. (1994), but not in yellow light, i.e. with blue and UV wavelengths removed, as used here. UV light breaks down calyculin (Matsunaga et al., 1991), and we found that plates containing calyculin lost effectiveness in proportion to the time they were pretreated with white light, but not with yellow light. Interestingly we found that the lag between transplantation onto calyculin and a detectable change in diameter was only a few hours

Table I. *Protein phosphatase and kinase inhibitors that neither inhibit elongation nor stimulate radial expansion of Arabidopsis roots*

Seven-day-old seedlings were transplanted onto plates containing inhibitors over a range of concentrations, and elongation and diameter of roots measured over the following 2 d did not differ from controls.

Compound	Chief Target[a]	K_i[b]	Highest Dose Tested
			μM
Phosphatase inhibitors			
Deltamethrin	PP2B	100 pM	20
Tautomycin	PP1, PP2A	200–400 pM	1[c]
Microcystin LR	PP1, PP2A	10–100 pM	30[c]
Kinase inhibitors			
KT 5720	PKA	56 nM	19
KT 5823	PKG	230 nM	20
KN 62	CMK II	0.9 μM	100

[a] PP, Protein phosphatase; PKA, cyclic AMP-dependent protein kinase; PKG, cyclic GMP-dependent protein kinase; CMKII, calcium/calmodulin kinase II. [b] Based on activity in dilute extracts. References are as follows: deltamethrin (Enan and Matsumura, 1992); tautomycin (MacKintosh and Klumpp, 1990); microcystin LR (MacKintosh et al., 1990); KT 5720, KT 5823, KN 62 (Hidaka and Kobayashi, 1993). [c] Elongation was inhibited by about 50% at highest dose, but root diameter was not increased.

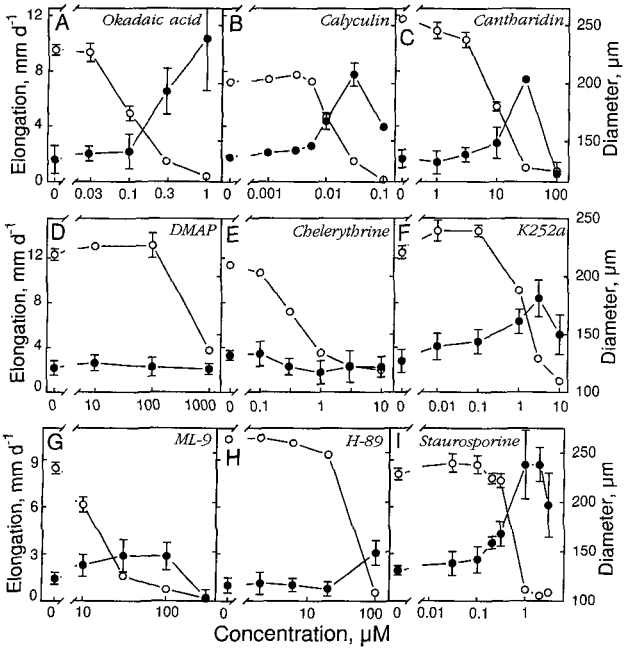

Figure 1. Dose-response curves for elongation rate and diameter of Arabidopsis roots in response to phosphatase or kinase inhibitors. Seven-day-old seedlings were transplanted onto plates containing inhibitors. The graphs plot elongation rate during the 2nd d after transfer (○) and diameter at the end of the 2nd d (●). A to C, Phosphatase inhibitors. D to I, Kinase inhibitors. For calyculin and cantharidin, symbols represent means ± SE for three replicate experiments; for the others, symbols represent the mean for a single experiment ±SE of three replicate plates for elongation and ±SD of 16 to 20 seedlings for diameter. Control elongation rates differ between the experiments for unknown reasons. Data for DMAP are from Baskin and Bivens (1995).

under white light (similar to the lag for cantharidin or staurosporine) compared with a full day under yellow light treatment (data not shown). The difference in lag times could be explained by supposing that modification of calyculin is required for biological activity, which occurs rapidly under white light by means of photochemistry, but it occurs slowly under yellow light, biochemically.

The six protein kinase inhibitors inhibited elongation in all cases, but three of them (DMAP, chelerythrine, and ML-9) did not stimulate radial expansion (Fig. 1, D, E, and G), and two (K252a and H-89) increased root diameter only slightly (Fig. 1, F and H). Similarly, DMAP has been reported to inhibit the elongation of bean epicotyls stimulated by gibberellin (Mizuno, 1994). However, one compound, staurosporine, caused significant radial expansion, comparable to that caused by the phosphatase inhibitors (Fig. 1I). For staurosporine, the thresholds for inhibiting elongation and for increasing root diameter were similar.

For all of the compounds shown in Figure 1, the inhibition of root elongation could in principle follow from the inhibition of cell division. If these compounds blocked cell division without affecting elongation, average cell length would increase; however, maximum cell length was greatly reduced compared with controls for all active inhibitors (not shown), implying that the cell elongation was directly affected by the compounds. All of the compounds that caused significant swelling, okadaic acid, cantharidin, calyculin, and staurosporine, did so in the region of elongation as well as in the meristem, although staurosporine caused more extensive swelling in the meristem than did the other compounds. Calyculin caused epidermal cells to separate, especially in the elongation zone (Smith et al., 1994), whereas staurosporine caused cells to separate in the interior tissues of the meristem (Fig. 3C). Furthermore, calyculin and okadaic acid each inhibited root hair growth at low nanomolar concentrations (Smith et al., 1994); however, cantharidin inhibited root hair growth only at similar concentrations that caused swelling, and staurosporine did not noticeably inhibit root hair growth. The difference in sensitivity to these inhibitors of elongation in root hairs versus the root proper is a further indication that tip growth and diffuse growth are mediated by divergent pathways (Emons et al., 1992).

To examine the relationship between the pathways affected by each type of inhibitor, we transplanted seedlings onto plates containing both cantharidin and staurosporine. When saturating levels of each compound were used, root elongation was inhibited and root diameter was stimulated to the same extent as with either compound alone (Table II). Similarly, when low levels of each compound were used, the response to the combination was essentially the average of the response to either alone during the 1st day of treatment (Table II) and also for the 2nd day, when the responses became more extreme (not shown). That the combination of inhibitors produces neither synergistic nor additive effects implies that they are acting in the same pathway for controlling morphology and probably at different steps.

Effects of Protein Phosphorylation Inhibitors on the Organization of Cortical Microtubules

To determine the basis for the altered morphology seen in roots treated with inhibitors, we examined microtubule arrays. We used sections embedded in removable butylmethyl methacrylate because this method is known to provide good localization of microtubules in most cells of the Arabidopsis root (Baskin et al., 1992, 1996). Seedlings were

Table II. *Effects on root elongation and diameter of combined treatment with a protein phosphatase inhibitor, cantharidin, and a protein kinase inhibitor, staurosporine*

Seedlings were transplanted onto inhibitors, root elongation rate was measured over the 1st d following transfer, and root diameter was measured at the end of the 1st d. The experiment was done three times and the data are the means ± SE of the three replicates.

Treatment	Elongation Rate	Diameter
	mm d⁻¹	*µm*
Control	9.2 ± 0.66	139 ± 3
Low levels:		
3 µM Cantharidin	7.88 ± 0.11	142 ± 4
0.17 µM Staurosporine	6.1 ± 0.23	178 ± 12
Both	6.61 ± 0.1	164 ± 7
High levels:		
30 µM Cantharidin	1.86 ± 0.3	205 ± 20
1 µM Staurosporine	1.68 ± 0.23	238 ± 18
Both	1.52 ± 0.28	220 ± 22

exposed to the inhibitors and fixed after 15 h (except for yellow light calyculin, for which 38 h was used) when the effects on expansion rates are approximately steady. In controls, microtubule arrays in cells at the beginning of the zone of rapid elongation were dense and mostly transverse to the long axis of the root (Fig. 2A). Microtubule cortical arrays were indistinguishable from controls in cells of roots treated with 1 μM chelerythrine (Fig. 2B) or 100 nM okadaic acid (Fig. 2C), which do not stimulate radial expansion.

However, microtubule arrays in cells of roots treated with 1 μM staurosporine (Fig. 2D), 30 μM cantharidin (Fig. 2E), or 170 nM (white light) calyculin (Fig. 2F) were notably disorganized. Calyculin, in addition to disorganizing cortical microtubules, also enhanced the nucleation of microtubules by the nuclear envelope, often including several prominent foci (Fig. 2F, inset). Microtubule disturbances seen for calyculin appeared to be the same whether plants were treated under white light (170 nM for 15 h) or yellow light (30 nM for 38 h); similarly, at 300 nM okadaic acid, a concentration that stimulated radial expansion, microtubules were disorganized and nucleation at the nuclear envelope was enhanced (not shown). For all treatments the abundance of cortical microtubules was approximately similar, as judged from similar exposure times being needed for photography. Disorganized cortical microtubules were found in all tissues and regions of

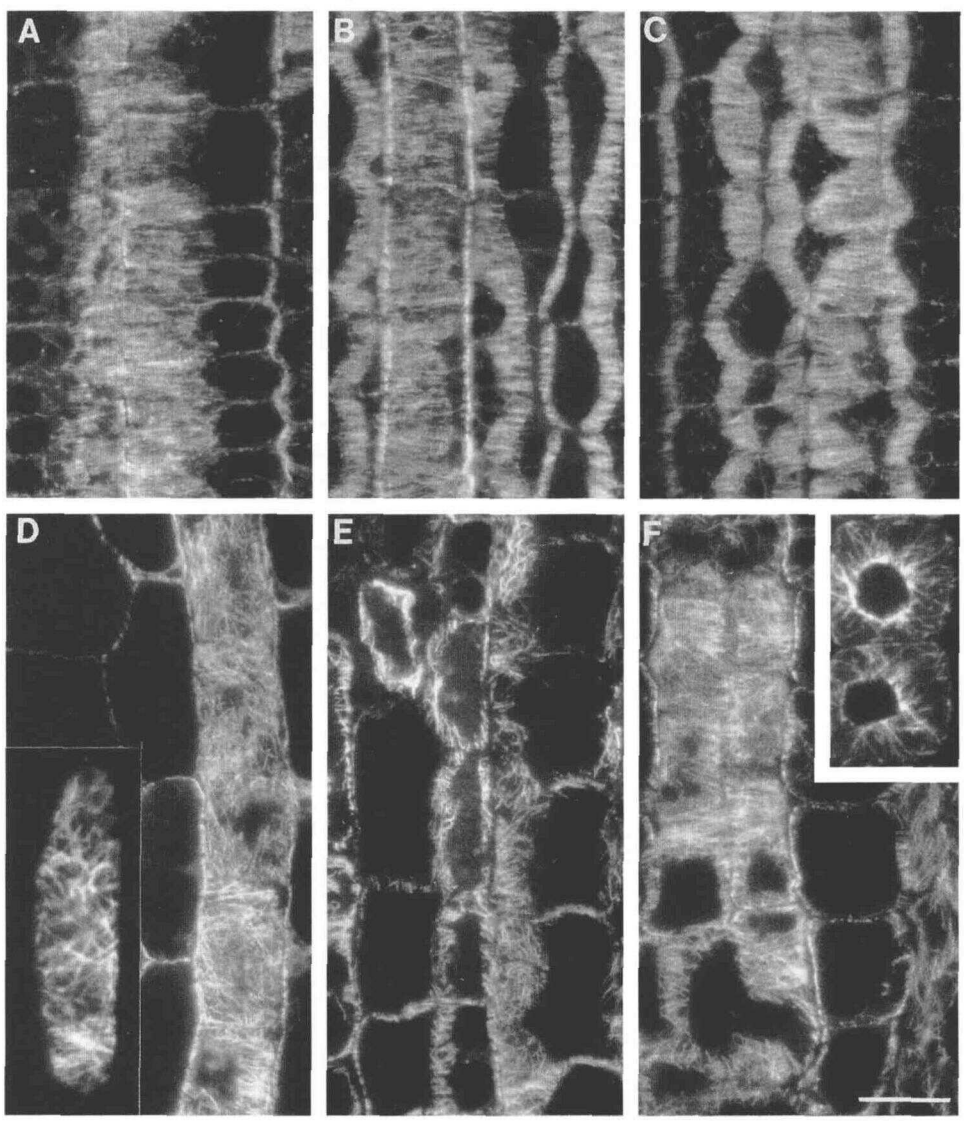

Figure 2. Localization of microtubules in Arabidopsis roots treated with protein phosphatase or kinase inhibitors for 15 h. *Images are of longitudinal sections passing through epidermis, cortex, or endodermis and show a region of the root near the proximal end of the zone of cell division where rapid expansion occurs.* A, Control; B, 1 μM chelerythrine; C, 100 nM okadaic acid; D, 1 μM staurosporine; inset, the cortical array of an epidermal cell at higher magnification for clarity; E, 30 μM cantharidin; F, 170 nM calyculin (white light treatment); inset, cells with enhanced microtubule nucleation at the nuclear *envelope, typical for this treatment.* Roots were fixed, embedded in butyl-methyl-methacrylate, sectioned, and stained with an anti-α-tubulin antibody. Magnification: A to F, ×708, bar = 15 μm; inset in D, ×1254; inset in F, ×1006.

the root, although the zone of cell division appeared less affected by calyculin or cantharidin compared with staurosporine. Disorganized arrays were present in mature cells as well as in those located just proximal to the zone of cell division, which is the region shown in Figure 2, where radial expansion is extensive. In some cells transverse arrays remained in whole or in part (Fig. 2F), whereas in others no vestige remained of the previous order (Fig. 2D, inset).

To determine whether the observed microtubule disorganization was accompanied by changes in microtubule stability, we tested the sensitivity of the microtubule arrays to two agents that cause depolymerization, cold and oryzalin. Low temperature shifts the equilibrium between soluble and polymerized tubulin strongly toward the soluble form (Gunning and Hardham, 1982). Oryzalin acts by binding to soluble tubulin dimers and preventing their polymerization onto microtubules (Hugdahl and Morejohn, 1993). Without soluble tubulin there can be no polymerization to balance the naturally occurring depolymerization; therefore, when microtubule arrays become more stable, having lower rates of depolymerization, they will persist longer in the presence of oryzalin. Seedlings were treated with calyculin, cantharidin, or staurosporine for 15 h and were then transplanted onto plates that were either prechilled on ice, or contained 1 μM oryzalin, which removes nearly all microtubules in this species (Baskin et al., 1994). When seedlings were fixed after 15 min on cold plates, control roots and those treated with inhibitors had each lost more than 50% of their cortical microtubules (not shown). Similarly, when seedlings were fixed after 30 min on oryzalin, control roots as well as those treated with inhibitors had only remnant microtubules and high background, which is typical of oryzalin treatment (Fig. 3). Results for staurosporine are shown (Fig. 3C), but results for cantharidin and calyculin were similar. When inhibitor-treated seedlings were transplanted onto DMSO in place of oryzalin for 30 min, the disorganized appearance of cortical arrays did not change (not shown). After only 15 min on oryzalin, about 50% of the microtubules remained in all treatments (not shown). Therefore, the disorganization of cortical microtubules caused by the protein phosphorylation inhibitors is not accompanied by a major change in microtubule stability.

Table III. *Effect of selected protein phosphatase and kinase inhibitors on nuclear density and mitotic index*

Roots were treated with inhibitors and processed for microscopy as described for Figure 2, and DAPI staining was used to localize nuclei. Interphase and mitotic nuclei in the apical 275 μm of the root (excluding the root cap) were counted in roughly median longitudinal sections, six to eight sections per root.

Treatment	n[a]	Nuclei[b]	Mitotic Nuclei[c]
Control	3	176 ± 21	2.04 ± 0.17
170 nM Calyculin	4	129 ± 9.2	1.92 ± 0.52
30 μM Cantharidin	4	157 ± 6.7	3.0 ± 0.44
1 μM Chelerythrine	4	199 ± 9.9	1.25 ± 0.21
1 μM Staurosporine	5	96 ± 12	0.51 ± 0.22

[a] Number of roots imaged in the section. [b] Average number ± SE of total nuclei [c] Percentage ± SE of nuclei judged to be in any phase of mitosis.

Effects of Protein Phosphorylation Inhibitors on Cell Division

In the slides prepared for examination of cortical arrays, all mitotic stages could be recognized in all inhibitors. Sometimes mitotic spindles and phragmoplasts stained weakly, but in contrast to cortical arrays, all types of mitotic array appeared to be organized normally. As a crude measure of cell division activity, we counted the percentage of nuclei in mitosis within the first 275 μm from the quiescent center (excluding root cap cells), where cell length remained short. The total number of nuclei decreased in calyculin, cantharidin, and especially staurosporine (Table III), indicating that the balance between cell division and cell expansion was shifted in favor of expansion. Conversely, the number of nuclei increased in chelerythrine, suggesting that this compound inhibited

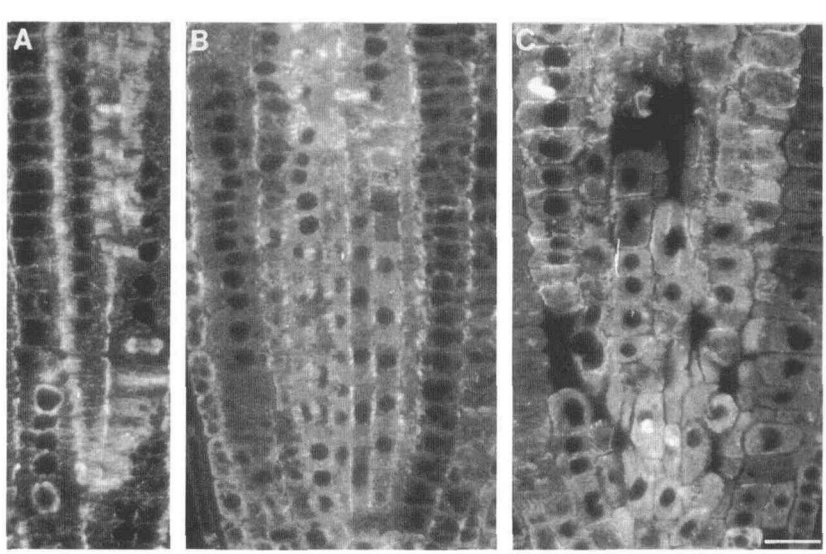

Figure 3. Sensitivity of microtubules in control or staurosporine-treated Arabidopsis roots to disruption by 1 μM oryzalin. Images are of longitudinal sections through the meristem. A, Control (transplanted onto 0.1% DMSO for 15 h and then again for 30 min). Note abundant cortical and mitotic arrays. B, Control, followed by oryzalin for 30 min. Note remnant microtubules and high background. C, Root treated with 1 μM staurosporine for 15 h (same as for Fig. 2) and then oryzalin for 30 min. Note remnant microtubules and high background, as well as swollen cells with irregular shapes and some gaps in the tissue. Magnification: A to C, ×511, bar = 15 μm.

meristematic cell expansion to a greater extent than cell division rate. We also counted mitotic nuclei. Although staurosporine significantly reduced the number of mitoses observed (Table III), no compound led to the striking increases or decreases in mitotic index seen when cells are strictly arrested at a particular cell cycle phase. This is consistent with studies of dividing plant cells in which phosphorylation inhibitors blocked the cell cycle at more than one phase (Hasezawa and Nagata, 1992; Katsuta and Shibaoka, 1992; Wolniak and Larsen, 1992; Zhang et al., 1992).

DISCUSSION

We have found that the elongation and shape of the Arabidopsis root is sensitive to the presence of several inhibitors of protein phosphorylation. Our earlier work showed that this was true for two protein phosphatase inhibitors (Smith et al., 1994), and here we extend that work to include another phosphatase inhibitor as well as a protein kinase inhibitor. We show further that the compounds that distort the morphology of the root also disorganize cortical microtubules. Although several authors have speculated that protein phosphorylation may play a role in organizing cortical microtubules and hence in morphogenesis (Cyr, 1994; Shibaoka, 1994), our results provide evidence that this is so.

Different Classes of Protein Phosphatases and Kinases

By choosing a range of inhibitors, we have attempted to gain some information about the class of activity involved. For protein phosphatases, four major classes have been defined based on substrate specificity and inhibitor sensitivity, and this delineation appears to be the same for plants as for fungi and animals (Smith and Walker, 1996). These classes are types 1, 2A, 2B, and 2C, and from our results it seems that either or both types 1 and 2A play a role in regulating root expansion and shape. The inhibitors okadaic acid, calyculin, and cantharidin are all known to be active against types 1 and 2A (Li and Casida, 1992; Smith and Walker, 1996). These three compounds are structurally unrelated, suggesting that the observed effects on growth did not arise from a nonspecific side reaction. Other inhibitors of types 1 and 2A phosphatases, microcystin (MacKintosh et al., 1990) and tautomycin (MacKintosh and Klumpp, 1990), inhibited elongation only partially (Table I), but these compounds may have been taken up poorly by the roots or may have been inactivated.

Unlike the rather small number of protein phosphatase classes, protein kinases come in many flavors. For our experiments we chose several inhibitors based on selectivity toward major types of kinases present in animal cells. On that basis our results implicate a protein kinase C-like activity in controlling elongation. Protein kinase C is inhibited selectively by chelerythrine (Herbert et al., 1990), and the K_i reported for this compound (0.6 μM) is similar to the concentration that inhibited elongation by 50%. Also, the reported K_is of many of the inhibitors used here against protein kinase C predict reasonably well their effect (or lack thereof) on elongation (Hidaka and Kobayashi, 1993). In agreement, a protein kinase C-like activity has recently been shown to help regulate the plasma membrane proton-ATPase, which is pivotal for elongation (Hoeven et al., 1996). Nevertheless, homologs of protein kinase C have not been cloned from plants, despite strenuous efforts, and it appears that the position occupied by protein kinase C in animal signal transduction is occupied in that of plants by protein kinases with distinct lineages (Stone and Walker, 1995). In plants there are many candidates for the functional analog of protein kinase C, but their sensitivity to the inhibitors used here has not been elucidated. Therefore, the indication from pharmacology of a protein kinase C-like activity regulating elongation cannot yet be applied to an identified biochemical pathway. The high activity of chelerythrine against elongation and its low cost may provide a tool with which to uncover the relevant protein kinase.

Separate Effects on Expansion Rate and Anisotropy

For plant morphogenesis, one can distinguish two features of the growth of cells: one is the rate of volumetric expansion, and the other is the direction of expansion. When growth rates are different in different directions, growth is said to be anisotropic. It is growth anisotropy, as opposed to the total rate of expansion, that is thought to be controlled by cortical microtubules (Green, 1980). In Arabidopsis roots, when microtubules are completely removed with oryzalin, the observed rates of elongation and radial expansion are consistent with an undiminished rate of volumetric expansion (Baskin and Bivens, 1995). Oryzalin causes a maximum radial expansion more than double the maximum seen here for any inhibitor, which indicates that the protein phosphorylation inhibitors not only reduced expansion anisotropy but also inhibited the overall rate of expansion. The reduction of the anisotropy and rate of expansion probably reflect the inhibition of distinct activities, because different protein kinase inhibitors stimulated radial expansion to different extents and because low doses of cantharidin or okadaic acid affected elongation but not radial expansion. The reduction of the degree of anisotropic expansion is plausibly explained by the disorganization of cortical microtubules.

Mechanisms for Microtubule Organization

The mechanism for organizing cortical microtubules is an unresolved problem for plant cell biology. It is useful to view the problem of this organization in the context of signal transduction (Cyr, 1994; Hush and Overall, 1996), in which three steps are traditionally recognized: perception, transduction, and response. To produce a group of microtubules all running in a similar direction, first, there must be a signal that specifies direction; second, that signal must be perceived by some cellular components and transmitted

to the microtubules; and third, the microtubules themselves must actually line up accordingly. Models have been proposed to explain each of these steps. The signal providing a polarity cue could be mechanical (Williamson, 1991; Cyr, 1994), electrical (Hush and Overall, 1996), or chemical (Shibaoka, 1994). The transducing mechanism is widely held to be some kind of transmembrane protein (complex) with an extracellular domain that "senses" the signal and a cytosolic domain that constrains microtubule behavior. Microtubule behavior could be modified by changing microtubule-microtubule interactions (Wymer and Lloyd, 1996), by rotating microtubules into correct alignment by a putative rotary motor protein (Cyr and Palevitz, 1995), or by selectively stabilizing those microtubules that happen by chance to polymerize at the correct angle (Wasteneys and Williamson, 1989). The loss of appropriate microtubule organization that we see could result from disruption at any of these levels.

Microtubule behavior is an attractive possibility because in animal cells the phosphorylation status of several microtubule-associated proteins has been found to alter microtubule dynamics and organization (Gurland and Gundersen, 1993; Ohta et al., 1993; Masson and Kreis, 1995; Ookata et al., 1995), and Sheldon and Wadsworth (1996) have shown directly in living cells that okadaic acid increases microtubule dynamics. In plant cells if the selective stabilization model is correct and organized cortical arrays depend on a difference in stability as a function of microtubule angle, then a treatment that obliterated this difference, e.g. by making all microtubules highly stable, would result in a randomized array. In fact, there is evidence from plant cells showing that protein phosphorylation can modify the dynamics of cortical microtubules. First, their cold stability can be changed by treatment with staurosporine or DMAP, increased for suspension-culture cells and decreased for protoplasts (Mizuno, 1992). Second, and more directly, fragments of the cortical array preserved on membrane ghosts from protoplasts are depolymerized by ATP but remain stable when ATP is given with staurosporine (Katsuta and Shibaoka, 1992). This indicates that a staurosporine-sensitive kinase may be partly responsible for maintaining the high rate of microtubule dynamics that are known to occur in the plant cortical array (Hush et al., 1994; Yuan et al., 1994). To see whether the randomized cortical arrays induced by the phosphorylation inhibitors were also hyperstabilized, we examined their behavior in the presence of cold or oryzalin. The disorganized arrays were removed just as quickly as were the organized arrays of untreated cells, which argues that the inhibitors have not appreciably altered the dynamics of microtubules.

Instead of acting on the response, i.e. on microtubule behavior, the inhibitors could be acting on the positional information signal or on its transduction. Evidence that this may be so can be gathered by considering where else disorganized microtubules are seen. Although the cortical array changes its organization in response to a wide variety of treatments, such as hormones, light, or other perturbations, these agents rarely induce random arrays. By contrast, random cortical arrays are seen in the cells of the quiescent center of the maize root (Baluška et al., 1992; Blancaflor and Hasenstein, 1995) and at the shoot apical meristem of several species of plants (Sakaguchi et al., 1988, 1990). The root quiescent center and the dome of the shoot meristem are both positions where cells presumably grow without a defined polarity and where there may be no directional signal made or interpreted. Additionally, random cortical microtubules are commonly seen in protoplasts (Shibaoka, 1994). Since most of the models for organizing cortical microtubules posit an extracellular signal that is perceived by transmembrane linkers (Williamson, 1991; Cyr, 1994; Shibaoka, 1994; Hush and Overall, 1996), the loss of microtubule organization in protoplasts can be explained by the inability to generate or interpret a uniform directional signal. That random microtubule arrays are also caused by protein phosphorylation inhibitors suggests that these inhibitors are acting on a process that generates a signal specifying cell polarity, or on a process by which that signal is transduced to specify microtubule organization.

Other investigations in which microtubules have been localized in plant cells treated with phosphorylation inhibitors, including staurosporine and calyculin, have not reported random cortical arrays (Hasezawa and Nagata, 1992; Katsuta and Shibaoka, 1992; Mizuno, 1992; Zhang et al., 1992). These studies were primarily focused on cell division, and all used tobacco tissue culture cells grown under conditions under which expansion is nearly isotropic. Their published micrographs show that the cortical array in control cells is often not well organized; therefore, effects on the organization of this array could have easily been missed. Furthermore, in this cell type, protein kinase inhibitors were found to enhance the stability to cold of the cortical array (Mizuno, 1992) and to slow down both formation and disappearance of the preprophase microtubule band (Katsuta and Shibaoka, 1992). These results suggest that the protein kinase inhibitors suppressed microtubule dynamics in the tobacco suspension-culture cells, which in turn may have hindered the rearrangement needed to produce random microtubules.

Nevertheless, to our knowledge the only other study to have examined the effects of any phosphorylation inhibitor on microtubules in a highly polarized plant cell type also did not find randomized cortical microtubules (Mizuno, 1994). In that study, which used segments from the azuki bean epicotyl, DMAP inhibited the stimulation of elongation by gibberellin, and in epidermal cells, in which microtubules were localized, DMAP caused cortical arrays to become longitudinal, regardless of the presence of gibberellin. In the Arabidopsis root, DMAP did inhibit elongation but did not cause radial expansion, so, like chelerythrine, we would not expect it to have disorganized microtubules. However, Mizuno (1994) stated that the results for DMAP were identical to those for staurosporine, which differs from the present findings. While this presumably reflects a difference among species or organ types, it is noteworthy that in the epicotyls, although longitudinally organized microtubules formed, they did not drive predominant radial expansion seen when longitudinal micro-

tubules form in azuki bean epicotyls treated with cytokinin (Shibaoka, 1974). Therefore, in the bean epicotyl, like the Arabidopsis root, protein phosphorylation inhibitors appear to disrupt the normal polarity-determining mechanisms of the organ. It remains for future work to elucidate the biochemical basis of the determination of polarity and its interpretation by cortical microtubules.

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